EPIDEMIOLOGICAL SURVEY FOR INCIDENCE OF SHIGELLOSIS AND HEPATITIS TO EVALUATE POTENTIAL FIELD TEST SITES FOR VACCINE TRIALS

PART 1 - SHIGELLOSIS

Final Report

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March, 1988

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Fredrick, Maryland 21701-5012.

Grant No. DAMD17-86-G-6026

Military Post 02149
Israel Defense Forces
Israel

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Military post 02149, Israel Defence Forces; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Military Post 02149, Israel Defence Forces, Israel	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-86-G-6026
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO
64758A	3S46 4758D 849	AB	021

Epidemiological Survey for Incidence of Shigellosis and Hepatitis to Evaluate Potential Field Test Sites for Vaccine Trials: Part 1: Shigellosis

12. PERSONAL AUTHOR(S): MANFRED S. GREEN, DANI COHEN, COLIN BLOCK, TZVI ROUACH

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
FINAL, Part I	FROM 6-1-86 TO 5-31-87	1988 March	29

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	DIARRHEA; DYSENTERY; EPIDEMIOLOGY; SEROLOGY; IMMUNOLOGY, BACTERIOLOGY; SHIGELLA VACCINES, RIA; HEPATITIS A.
06	06		
06	13		

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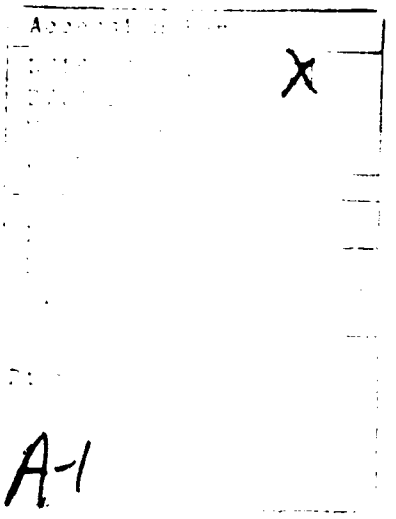
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mrs. Virginia M. Miller	301 - 663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

SUMMARY

The aim of this study was to determine the incidence of shigellosis in selected units in the IDF and to evaluate the associa- homologous Shigella and the occurrence of disease. In this study, soldiers in five units were followed prospectively for the occurrence of diarrheal disease. Blood samples were taken at the beginning and end of the study period. In four of the units, both blood and stool samples were taken from cases of diarrhea at the time of the event. Serologic response was determined by enzyme linked immunosorbent assay (ELISA). Epidemic and sporadic morbidity due to Shigella organisms was identified using bacteriological and serological diagnostic methods. The presence or absence of specific antibodies against Shigella at the onset of disease, were compared in cases and healthy controls from the same units. The presence of specific antibodies of the IgG class was a significant marker of protection against shigellosis due to homologous organisms.



FOREWORD

The development of new vaccines against shigellosis and hepatitis A, has necessitated the planning and execution of clinical trials of the vaccines. In order to improve the efficiency of these trials, and in order to evaluate their efficacy, it was felt that serologic markers of evidence of previous disease and of susceptibility to future disease should be studied. The current studies of the seroepidemiology of shigellosis and hepatitis A were carried out as a collaborative project between the Israel Defence Force Medical Corps and the Walter Reed Army Institute of Research. For the protection of human subjects, the investigator(s) have adhered to policies of applicable Federal Law 45CFR46. Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organisations.

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1.0 BACKGROUND

Shigellosis is responsible for much of the diarrheal disease suffered by soldiers serving in the Middle East (6,13) and in other operational areas such as Vietnam (21). Diarrhea continues to be a major factor in determining the operational capability of military units (21) and to engage much of their preventive medicine resources (6). In military populations, a vaccine may be the only reliable means of preventing epidemics of shigellosis (13) and recently developed vaccines appear promising (20).

One aspect considered to be particularly important in the context of future vaccine trials was to evaluate the level of natural immunity against shigellosis in populations amongst whom the vaccine will be tried. We have previously confirmed that serology can be used as a sensitive and specific index of morbidity due to Shigella (4). Although in the convalescent stage of shigellosis there is a rise in serum anti-LPS antibodies (3,12,19) their role in the immune process is not clear. Immunity against shigellosis is currently believed to be due to local secretory antibodies (15) but both cell-mediated immunity and humoral factors may also be important in the mechanism of protection (1,15,17).

In challenge studies conducted by DuPont et al (8) no association was found between pre-challenge hemagglutinins and protection of volunteers against disease caused by virulent S.flexneri 2a. Similar findings were reported in early studies of killed Shigella vaccines (11,14). In an animal model however, it has been shown that immunity against shigellosis can be transferred among rabbits using serum anti-outer membrane protein antibodies elicited by virulent strain of S.flexneri (1). The identity of the Shigella antigens which stimulate production of protective antibodies is still not clear. Both membrane lipopolysaccharides (LPS) (16) and outer membrane proteins (1) have been suggested as possible candidates.

In previous case-control studies, we found that serum levels of some or all of the specific anti-LPS immunoglobulins are associated with protection against naturally acquired disease (4). We found that the levels of anti-LPS antibodies present in subjects before or at the onset of an outbreak, particularly those in the non-IgM fraction as determined by passive HA, and the IgG fraction as determined by ELISA, were associated with significant protection against the disease. This protection was demonstrated both in the significantly lower GMTs found among cases compared with controls, and in the high relative risk of disease found among those with "low" pre-existing antibody levels as compared with those with higher levels. We found that pre-existing levels of anti-LPS antibodies indicate group specific protection against shigellosis, corroborating data previously reported on sero-type and sero-group specific immunity conferred by the O polysaccharide chain of LPS (10).

The present study was designed to test the findings of the case-control studies in a prospective mode.

2.0 SPECIFIC AIMS

Objectives

1. To evaluate the usefulness of specific immunoglobulin sub-fractions in defining immune status against Shigella under natural conditions of exposure.

2. To evaluate the incidence of shigellosis in military populations serving under field conditions.

3.0 METHODS

3.1 Study design

Prospective studies.

3.2 Study population

The shigellosis study was conducted in five bases during the summer months from June 1986 to November 1986. Continuous surveillance was maintained in several IDF units for cases of diarrhea. The study populations included male soldiers aged 18-20. Each unit was followed for approximately two months.

Criteria for diagnosing shigellosis

Any subject complaining of diarrhea with either isolation of Shigella organisms in the stool culture or significant antibody response against Shigella LPS.

Questionnaire (see appendix)

Data on presence or absence of symptoms or signs of disease, date of onset, and a description of the feces were obtained from all cases of diarrhea presenting at the unit clinic and 10-14 days later.

3.4 LABORATORY STUDIES

Bacteriology

Stool samples were obtained at the time of the visit to the unit medical clinic. On receipt, specimens were planted immediately on MacConkey agar and SS agar. In parallel, swabs with fecal matter were introduced in buffered glycerol transport medium and GN enrichment broth. Specimens were kept cool in polystyrene boxes until arrival at the laboratory. At the central laboratory, additional plating of samples from transport and enrichment media was made on MacConkey and SS agars. Subcultures were made from buffered glycerol transport medium to Campylobacter blood agar (with Preston selective suplement). After overnight incubation at 35-37°C representatives of all non-lactose fermenting colonies on MacConkey and SS agars were identified by routine morphological, biochemical and serological testing. After incubation at 42°C, for 48 hours in a micro-aerophilic environment, suggestive colonies on the campylobacter medium were selected for identification on basis of bacterial morphology and oxidase positivity. Enteric pathogens identified were submitted to the Reference Laboratories of the Israel Ministry of Health for confirmation and further identification.

Serology

Blood samples were taken from subjects at the begining and end of the training period in the field. Additional paired sera were obtained from cases of diarrhea at the acute and convalescent stages of the disease. Sera were separated and aliquots frozen at -20°C until used for titration. Serological tests were carried out in duplicate using the Enzyme-Linked Immunosorbent Assay (ELISA).

ELISA was performed basically using the method of Engval and Perlman (Engval, Perlman 1971) (10) adapted for polystyrene microtitration plates (Nunc) (9). Some modifications concerning the conditions and the length of the incubation periods were introduced (Chung R. Personal communication 1986). Phenol water extracted LPS from clinical isolates of S.sonnei form 1 and S.flexneri 2a, was suspended for coating the solid phase.

The optimal LPS antigen concentration was found to be 10mcg/ml, the concentration used throughout the study. An 0.05 M carbonate buffer, pH 9.6 was used for coating at 37°C for 1h. In addition the microtiter plates were incubated for 1 hour at 37°C with 0,05 M PBS-test buffer supplemented with casein and bovine serum albumin (both 5g/l) in order to competitively prevent non-specific attachment of the conjugated anti-immunoglobulins.
The wells were then washed twice in PBS-Tween 20 washing solution. The sera at a dilution of 1:100 in PBS-test buffer, were

added to the wells and incubated overnight at room temperature. After four further washings, anti-IgM, anti-IgG, or anti-IgA conjugated to alkaline phosphatase (Perry Lab.) and diluted 1:500 were added to the wells and incubated overnight at room temperature. Four washings in the same solution preceded the addition of the enzyme substrate solution, paranitrophenylphosphate (1mg/ml) in diethanolamine buffer, pH 9.8. The enzyme reaction was stopped by the addition of 3 M NaOH to the wells after incubation for 15 or 30 minutes at room temperature. The enzymatic activity of alkaline phosphatase was expressed in absorbance values determined at 405 nm using an automatic ELISA reader (Kontron, SLT). The results were expressed as optical density values of the 1:100 dilutions and a significant antibody response was defined as a 2.5 fold or greater increase in the optical density value between the two bleedings.. Control sera were included in every two microtitration plates in each of the assays. Inter- and intra-assay adjustments were made where necessary.

We defined "pre-existing" anti-LPS antibodies as an ELISA IgG optical density of 0.3 or more at 1: 100 dilution of sera.

3.5 Statistical analysis

For the 2x2 tables risk ratios were computed as estimates of the relative risks of disease according to antibody status. Tests of significance were based on Fisher's exact test.

4.0 RESULTS

Characteristics of the study population are shown in Table 1. Two measures of diarrhea are given - clinic visits and recalled diarrhea by questionnaire at the end of the study period. The distribution of shigella isolates is given in Table 2. They were equally distributed between S.sonnei, S.flexneri, and S.boydii.

Shigellas were isolated at the four study bases where cultures were performed (see Table 2). Cases of shigellosis (diagnosed either by culture or serology) occurred in significant numbers in three of the five study sites (Bases B1, S and N - see Tables 1, 2)

Antibody response to S.sonnei and S.flexneri in those with paired sera is given in Tables 3 and 4. Rates of diarrheal events and cases of shigellosis are presented in table 5. Recalled diarrhea by baseline IgG against S.sonnei and S.flexneri is given in Tables 6 and 7. Shigellosis by baseline IgG against the homologous antigen is given in Tables 8 and 9. Test for specificity - shigellosis by baseline levels of heterologous antigens are given in Tables 10 and 11.

5.0 DISCUSSION

This prospective investigation has confirmed the association found in our previous case-control studies (4) between the presence of anti-shigella LPS and protection against naturally acquired shigellosis. Anti-shigella LPS IgG present before exposure was associated with a significantly reduced risk of developing clinically evident shigellosis.

We found that the levels of anti-LPS antibodies of IgG fraction as determined by ELISA, present in subjects before exposure to field conditions were associated with significant protection against the disease. This protection was demonstrated by the high relative risk of disease found among those with "low" pre-existing antibody levels as compared with those with higher levels. We found that pre-existing levels of anti-LPS antibodies indicate group specific protection against shigellosis, corroborating data previously reported on sero-type and sero-group specific immunity conferred by the O polysaccharide chain of LPS (16).

There are several possible explanations for the findings of the case-control and prospective studies that the association with protection against the disease lies mainly in the HA non-IgM fraction HA and the ELISA IgG fraction. The most likely is that high levels of serum IgG persist longer after natural exposure than for those of the other sub-fractions. This is in agreement with the view that development of natural immunity against Shigella sp. in endemic regions is a result of frequent natural boosters (7) which may lead to secondary responses and concomitant increases in the IgG fraction. It is also possible that, while secretory IgA antibodies play the major part in prevention of the disease, serum levels of IgA only partially reflect intestinal IgA concentrations. There may even be evidence for an active role for IgG in protection against shigellosis. For example, in studies on meningococci, IgG was found to be much more effective than IgM in antibody dependent cell-mediated antibacterial activity (17), a mechanism which has been demonstrated to be efficient in vitro against enteric pathogens like S.flexneri (18).

Our findings are supported by data recently reported by Black et al (2), where the presence of specific serum IgA and IgG antibodies, prior to experimental challenge with virulent S.sonnei, was correlated with protection against illness.

Shigellosis occurred, as predicted, at all the five bases chosen for the study, with high incidence rates being recorded at three of them. This information suggests that these same bases should be suitable as sites for future vaccine trials. Remarkable differences were found in the incidence of diarrhea recalled by the soldiers at the end of the study period, as compared with the in-

cidence of visits to the clinic for diarrhea and possibly suggests a high incidence of relatively mild diarrhea.

6.0 CONCLUSIONS

The absence of antibodies to the specific *Shigella* is strongly associated with susceptibility to the disease and may be used to identify people at an increased risk of shigellosis than in the general population.

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8.0 ACKNOWLEDGEMENTS

We wish to acknowledge the support of our collaborators at WRAIR, Col Edmund Tramont, Dr Samuel Formal, Lt Col George Lowell and other members of the staff at WRAIR for their generous assistance in all aspects of this work. We much appreciate the assistance of Dr Anna Johnson-Winegar of the USAMRDC, in expediting the adminstrative details of the grant so effectively. Finally we thank our consultants in Israel, Profs I Ofek and T Schwarz of the University of Tel Aviv for their contributions to the scientific aspects of the study.

9.0 TABLES

TABLE 1

Characteristics of the study population

	No of Soldiers (%)				
Unit	B1	B4	B9	N	S
No admitted to the study	293	212	240	141	306
Recalled diarrheal episodes (questionnaire only*)	124	38	94	62	72
Paired blood samples available	193 (65.8)	107 (50.4)	169 (70.4)	74 (52.4)	137 (44.7)
Clinic visits for diarrhea	23	35	19	ND	22
Stool cultures available	23	35	19	ND	22
Shigella sp	12	1	1	ND	22
Salmonella sp	0	0	0	0	0
Campylobacter sp	0	1	0	0	0

* Obtained at the end of the follow-up period

TABLE 2

Relative distribution of Shigella isolates at the various units*

Unit	S.sonnei	S.flexneri	S.boydii	S.dysenteriae	Total
B1	10	2	0	0	12
B4	0	2	0	0	2
B9	0	1	0	0	1
S	1	6	11	0	18
Total	11	11	11	0	33

* There was no active surveillance for cases at base N

TABLE 3

Antibody response* to S.sonnei LPS in symptomatic and asymptomatic subjects during the follow up period (recalled diarrhea for subjects with paired sera)

Unit	Recalled diarrhea+	No	Antibody response against S.sonnei LPS			
			IgM	IgG	IgA	Combined (%)
B1	Yes	124	9	20	35	38 (30.6)
	No	61	3	7	11	13 (4.5)
B4	Yes	38	0	0	1	1 (2.6)
	No	19	0	0	0	0
B9	Yes	94	1	3	10	11 (11.7)
	No	66	1	3	6	9 (13.7)
N	Yes	62	0	3	3	4 (6.5)
	No	9	0	0	0	0
S	Yes	72	1	2	6	9 (12.5)
	No	64	0	0	9	9 (14.1)

* 2.5 fold or greater increase in the ELISA OD measured at 1:100 dilution of sera

+ Information obtained by questionnaires administered to subjects at the end of the follow up period

TABLE 4

Antibody response* to S.flexneri 2a LPS in symptomatic and asymptomatic subjects during the follow up period (recalled diarrhea for subjects with paired sera)

Unit	Recalled diarrhea+	No	Antibody response against S.flexneri 2a LPS			
			IgM	IgG	IgA	Combined (%)
B1	Yes	124	2	2	2	2 (1.6)
	No	61	0	1	1	1 (1.6)
B4	Yes	38	1	1	2	3 (7.9)
	No	19	0	1	0	1 (5.3)
B9	Yes	94	0	4	2	6 (6.4)
	No	66	0	1	7	8 (12.1)
N	Yes	62	0	31	29	42 (67.7)
	No	9	0	4	4	6 (66.6)
S	Yes	72	3	3	6	9 (12.5)
	No	64	0	1	5	6 (9.4)

* 2.5 fold or greater increase in the ELISA OD measured at 1:100 dilution of sera

+ Information obtained by questionnaires administered to subjects at the end of the follow up period

TABLE 5

Rates of diarrhea and shigellosis for each base

Unit	n	Recalled diarrhea	Clinic visits for diarrhea	Shigellosis**
		No. (rate/100/mo)		
B1	193	124 (32.1)	23 (5.9)	42 (10.9)
B4	107	38 (17.7)	35 (16.3)	4 (1.9)
B9	169	94 (27.8)	19 (5.6)	17 (5.0)
N*	74	62 (41.9)	ND	46 (31.2)
S	137	72 (26.3)	22 (8.0)	29 (10.6)
Total	680	390	99	151

* There was no active surveillance for cases at base N

** Diagnosis by culture, serology or both

TABLE 6

Recalled diarrhea by baseline IgG antibody levels against S.sonnei LPS

	OPTICAL DENSITY*		
	≤ 0.3	> 0.3	Total
Diarrhea	82 (65.0%)	42 (71.1%)	124 (67.0%)
No diarrhea	44 (34.9%)	17 (28.8%)	61 (32.0%)
Total	126	59	185
	RR = 0.91	Fisher's exact p = 0.26	

* At a serum dilution of 1:100

TABLE 7

Recalled diarrhea by baseline IgG antibody levels against S.flexneri 2a LPS

	OPTICAL DENSITY*		
	≤ 0.3	> 0.3	Total
No diarrhea	4 (10.8%)	5 (14.7%)	9 (12.6%)
Diarrhea	33 (89.1%)	29 (85.2%)	62 (87.3%)
Total	37	34	71

RR = 1.04 Fisher's exact p = 0.44

*At a serum dilution of 1:100

TABLE 8

Shigellosis* due to S.sonnei by baseline IgG antibody levels against S.sonnei LPS

	OPTICAL DENSITY**		
	≤ 0.3	> 0.3	Total
Shigellosis (S.sonnei)	33 (25.0%)	6 (9.8%)	39
No shigellosis (S.sonnei)	99 (75.0%)	55 (90.2%)	154
Total	132	61	193

RR = 2.5 Fisher's exact p = 0.019

* Recalled diarrhea with positive stool culture for S.sonnei or significant antibody response in at least one of the immunoglobulin fractions

** At a serum dilution of 1:100

TABLE 9

Shigellosis* due to S. flexneri 2a by baseline IgG antibody levels against S.flexneri 2a LPS

	OPTICAL DENSITY**		
	≤ 0.3	> 0.3	Total
Shigellosis (S.flexneri)	21 (53.8%)	9 (25.7%)	30 (40.4%)
No shigellosis (S.flexneri)	18 (46.1%)	26 (74.2%)	44 (59.4%)
Total	39	35	74

RR = 2.1 Fisher's exact p = 0.012

* Recalled diarrhea with positive stool culture for S.flexneri 2a *or significant antibody response* in at least one of the immunoglobulin fractions

** At a serum dilution of 1:100

TABLE 10

S.sonnei shigellosis by baseline IgG antibody levels against S.flexneri 2a LPS (heterologous antigen)

	OPTICAL DENSITY*		
	≤ 0.3	> 0.3	Total
Shigellosis (S.sonnei)	22 (24.2%)	14 (14.0)	36 (18.8%)
No shigellosis (S.sonnei)	69 (75.8%)	86 (86.0%)	155 (81.1%)
Total	91	100	191

RR = 1.72 Fisher's exact p = 0.095

* At a serum dilution of 1:100

TABLE 11

S. flexneri 2a shigellosis by baseline IgG antibody levels against S.sonnei LPS (heterologous antigen)

	OPTICAL DENSITY*		
	≤ 0.3	> 0.3	Total
Shigellosis (S.flexneri 2a)	21 (37.5%)	9 (50.0%)	30 (40.5%)
No shigellosis (S.flexneri 2a)	35 (62.5%)	9 (50.0%)	44 (59.4%)
Total	56	18	74

RR = 0.75 Fisher's exact p = 0.25

* At a serum dilution of 1:100

10.0 QUESTIONNAIRE

Computer code

Number _____

Date _____

Name _____ I.D. _____

Military unit number _____

1. Type of service: 1. Compulsory 2. Permanent
   3. Reserves

2. Dining room: 1. Officers 2. Recruits 3. Joint
   4. Do not eat at base

3. Do you presently suffer from:

   Diarrhea 1. Yes 2. No

   Abdominal pain 1. Yes 2. No

   Nausea 1. Yes 2. No

   Vomiting 1. Yes 2. No

   Fever 1. Yes 2. No

4. Were you hospitalized? 1. Yes 2. No

5. Did you receive an infusion? 1. Yes 2. No

6. Date on which the diarrhea started _____

7. How many times did you have a bowel action _____

8. Did you suffer from urgency 1. Very much 2. Mildly
   3. Not at all

9. What was the consistency of the stools?
   1. Liquid 2. Soft 3. Normal

10. Did you see blood in the stools? 1. Yes 2. No

11. How many attacks of diarrhea of a similar nature did you have during the past year? _____

12. How many cigarettes do you smoke per day? _____

Part 2 - Demographic information

1. Age
2. Sex
3. Country of origin
4. Years of education

Part 3 - Laboratory results

1. Was a first stool sample taken? 1. Yes 2. No
2. Was a second stool sample taken? 1. Yes 2. No
3. Was a first blood sample taken? 1. Yes 2. No

Date _____

4. Was a second blood sample taken? 1. Yes 2. No

Date _____

Results:

First stool sample:

1. Shigella: 1. Negative 2. Dysenteriae 3. Flexneri 4. Boydii 5. Sonnei
2. Salmonella: 1. Negative 2. A 3. B 4. C 5. D 6. E
3. Campylobacter: 1. Negative 2.

SEROLOGY RESULTS

Antigen - S. sonnei

	First blood	Second blood
ELISA - IgM	_____	_____
ELISA - IgG	_____	_____
ELISA - IgA	_____	_____

Antigen - S. flexneri

	First blood	Second blood
ELISA - IgM	_____	_____
ELISA - IgG	_____	_____
ELISA - IgA	_____	_____